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Molecular identification of Fusarium spp causing wilt on *Araucaria araucana* and its management

Komal Ambreen, Anjum Faraz*, Nabeeha Aslam khan and Nasir Ahmad Khan

Department of Plant Pathology, University of Agriculture, University Road, Faisalabad, Pakistan
*Correspondent author's e-mail: faraz2537@gmail.com

Five sampling sites of evergreen conifer *Araucaria araucana* subjected to decline symptoms were randomly selected across the Punjab province, Pakistan. The declined plants with needle discoloration, chlorosis, drying, and partial to complete shoot wilt symptoms were sampled. The estimated highest disease incidence (DI) and disease severity (DS) was 51.8% and 70%, respectively. The most frequently obtained fungal isolates were identified as *Fusarium* sp. on a morphological basis and Koch's postulates were fulfilled. Once after the confirmation of the pathogen, one representative isolate was selected and sequenced for accurate identification. The characterized isolate showed 100% similarity with *F. solani*. Different bio-control agents, Systemic acquired resistance, and antifungal chemicals were integrated to manage the diseases effectively. The fungal antagonists, including *Aspergillus niger* and *Trichoderma harzianum*, were evaluated for their antifungal potential. *A. niger* and *T. harzianum* showed 71.1% and 55.2% mycelial inhibition, respectively. Among four tested fungicides (Aliette, Topsin M, Tiger, and Amistar), Tiger proved to be the most effective, with 88.8% mycelial suppression over control. For SAR, Salicylic acid (SA) was sprayed @ 1mM, and treated plants showed disease severity of up to 10% compared to fungicide-treated plants. This is 1st comprehensive study on *Fusarium solani* as the etiological agent of *A. araucana* and its management from Punjab, Pakistan.

Keywords: Evergreen conifer, dioecious, disease assessment, morphogenic, fungal antagonists, antifungal.

INTRODUCTION

Araucaria araucana (monkey puzzle or Chile pine), native to Chile and Argentina, is a pyramid-shaped, evergreen conifer that grows up to 50 m high with a stem diameter of 2 m. It is a dioecious tree; the male cone is cylindrical, and 15cm long, and the female cone is globular with a diameter of 20 cm. The tree is unique in its foliage beauty; the leaves are glossy, dark green, and stiff, covering the whole branch and overlapping each other (Aslam *et al*., 2013; Gehrig *et al*., 2009; Veblen and Delmastro, 1976; Veblen *et al*., 1995). The genus Araucaria is comprised of 19 species that are found in the northern hemisphere (Nimsch, 2011). *Fusarium* and *Botryospharia* species have been reported from various countries associated with Araucaria (Bullians *et al*., 2007).

The genus Fusarium belongs to Phylum Ascomycota and is considered among the most devastating fungal pathogens globally (Nelson, 1983). In Pakistan, Fusarium was reported as a pathogen from northern Pakistan in 1994. Literature provides strong evidence about *Fusarium solani* as the aggressive pathogen of several tree and plant species under different environmental conditions. However, humidity and temperature play a significant role in the growth, development, and germination of the *Fusarium* species (Marin *et al*., 1996). The fungus is particularly involved in tree dieback (Nelson, 1981**).** The fungus produces hard resting structures such as chlamydospores and micro and macroconidia. The fungus can survive in the soil as Chlamydospores for 10 to 20 years and up to 6 years without a host plant (Kraft, 1994).

Trichoderma and Aspergillus are important fungal biocontrol agents (Fravel *et al*., 2003). *Trichoderma harzianum* is an effective antagonist against *Fusarium* spp. (Malik and Dawar, 2003; Sharma, 2011). Salicylic acid plays an important role in inducing resistance against *Fusarium* spp. (Mandal *et al*., 2009; Yousif, 2017; Jendoubi *et al*., 2015). Tebuconazole (Tiger) and Thiophanate methyl (Topsin M) are the most effective chemicals against the Fusarium spp. Penconazole, Benomyl, Carbendazim, and Captan are effective fungicides in the suppression of Fusarium species (Desai *et al*., 2002 and

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Christian *et al*., 2007; Dwivedai *et al*., 1995; Kang *et al*., 2001).

A. Araucana is an integral part of Pakistan's indoor and outdoor landscape. Although few preliminary reports are available on diseases of perennial ornamental plants and their management, this area still needs to be explored. Recently, an emerging disease of Araucaria was observed in the vicinity of Faisalabad. So, the research was planned to determine the cause of declining Araucaria plants and to determine disease management strategies to overcome the problem.

MATERIALS AND METHODS

Five sampling districts Kasur,Lahore,Faisalabad, Rawalpindi, and Islamabad, were selected in Punjab to estimate disease incidence (DI) and severity (DS). For this purpose, commercial nurseries, avenues, parks, universities, and educational institutes from these sampling sites were randomly sampled. Fifty plants were randomly selected from each site to record the Percent DI and DS. Samples comprising symptomatic tissues and healthy tissues were taken and submitted to Fungal Molecular Biology (FMB) Laboratory. The roots and leaf samples were rinsed under tap water, cut into smaller pieces (3-4 mm), superficially sterilized in 2% sodium hypochlorite solution for 30 seconds, washed in sterile water, and removed from excessive moisture. Samples were processed on Potato Dextrose Agar (PDA; 15g/L Agar, 20g/L Dextrose, 20g/L potato starch, and 1000 mL Water) for the isolation of pathogenic fungal isolate(s). After 22-24 hours of incubation, fugal colonies that appeared on inoculated plates were picked from the marginal tips of growing hyphae, subcultured, and identified microscopically. Most frequently obtained fungal isolate(s) were purified by single sporing and preserved in Agar slants for future experimentation. Representative fungal isolate(s) was identified on a morphogenomic basis. The physiological and microscopic study of the representative isolate(s) was grown on PDA at 25^{o}C for six days at P^{H} 5.6 with 12 hours of photoperiod. The macroscopic characteristics include colony shape and colour on PDA. Microscopic observations, including the type of mycelium and spore(s) produced, spore shape, and colour were recorded at 10X, 20X, and 40X. For micro and macroconidia, 15 measurements of each structure were made at 40X. The purified fungal isolate(s) was submitted to the Fmb-cc-UAF acc. No. To check their virulence, one-year-old healthy *A. Araucana* plants were inoculated in the greenhouse. The pure fungal isolate was multiplied on Potato Dextrose Broth (PDB), sprayed employing foliar application (10% of the liquid broth @ 25ml), and soil drenching as well and allowed for symptom development. The disease severity data were recorded every week up to the 16th week. An integrated approach comprised of antagonists, Systemic Acquired Resistance (SAR: Salicylic Acid) chemicals and fungicides was adopted for disease management. The antagonistic potential of *Aspergillus niger* against a pathogenic isolate of *Fusarium solani* @ 25%, 50%, 75%, and 100% PDB was determined under controlled conditions by poison food technique, and Percent growth inhibition (PGI) was recorded at 48, 72 and 96 hours. The dual culture method was used to study the antagonistic potential of *Trichoderma harzianum* against the pathogen, and data regarding PGI was taken on the 3rd, 5^{th,} and 7th days. *In vitro* efficacy of Fosetyl aluminum (Aliette), Thiophanate methyl (Topsin M), Tebuconzole (Tiger), and Azoxystrobin + Difenoconazole (Amistar) against pathogenic fungal isolate @ 150, 200 and 250 ppm was determined by poison food technique and PGI was recorded at 48, 72 and 96 hours.

PGI was calculated as follows:

$$PGI=(C-T/C)\ 100$$

Where, C=Growth of pathogenic fungus in the control plate, T=Growth of the pathogenic fungus in the presence of antagonists and fungicides

Two fungicides (Topsin-M and Tiger) were selected based on their efficacy obtained during lab experiments for the greenhouse management of the disease. Healthy plants were inoculated with a 10% broth solution of *F. solani*, and on 4th day of post-inoculation, fungicides were sprayed on leaves and soil drenched, and disease severity data were recorded for up to four weeks. Plants of *A. araucana* was sprayed with a 1mM solution of Salicylic Acid (SA) to induce systemic resistance. The fungal inoculum was sprayed on the SA-treated plants after one week. Disease severity was observed for up to four weeks.

RESULTS

The inoculated plants produced symptoms (needle discoloration and partial shoot wilting) similar to those observed in surveys. Needle discoloration and drying of branches with partial to complete shoot wilting of the infected plants was commonly observed from most surveyed locations. The highest disease incidence of 51.8% and disease severity of 70% was noted in Pattoki (Kasur) Table 1. Maximum disease severity of 59% was observed at the end of the 16th week of post-inoculation. The pathogen was identified on a morphogenomic basis as Fusarium solani. The pathogen produced abundant white to creamy sparse mycelium with septations and abundant creamy to green Sporodochia on PDA. The fungus produces numerous macroconidia ranging from 7.50-16.25µm, and microconidia were 5 to 6.75µm and chlamydospores. Microconidia were aseptate and oval-shaped, while the macroconidia possessed 5-6 septations and were slightly curved with thin walls. Hence, Fusarium solani was proved as the etiological agent of the declining A. araucana based on the completion of Koch's postulates.

A. niger and *T. harzianum* have a significant role in the mycelial suppression of pathogenic *F. solani*. Antagonists (*A.*

niger and *T. harzianum*) used in this study were effective, with 71.1% and 55.2% percent inhibition, respectively. All the fungicides selected for *In vitro* study showed good antifungal potential against *Fusarium solani*, the cause of Araucaria decline in Punjab, Pakistan. However, Tebuconazole (Tiger) @ 250 ppm possessed the highest antifungal potential with a mean PGI of 88.8% over control, followed by Thiophanate methyl (Topsin M) 84.4%, Fostyle Aluminum (Aliette) 81.1% and Azoxystrobin+ Difenoconazole (Amistar) 77.7% respectively.

The Topsin-M (Thiophanate methyl) and Tiger (Tebuconazole) @ 250ppm were effective fungicides under a controlled environment hence, selected to determine their efficacy in the greenhouse. The results indicated that Tiger @ 250ppm exhibited minimum disease severity of 5%, 10.5%, 11.25%, and 20.8% after the 1st, 2nd and 3rd, and 4th weekly application of fungicides, respectively. However, SA was acid was most effective compared to Tiger (Tebuconazole) and Topsin-M (Thiophanate methyl) under greenhouse conditions. Applying SA reduced the disease severity and enhanced the physical appearance (Lush green) and vigour of the inoculated plants. Salicylic acid restricted disease severity to 10% at the end of 4th week of application as compared to other fungicidal treatments. Furthermore, Tebuconazole (Tiger) also promised effective disease management in laboratory and greenhouse conditions.

Table 1. Disease documentation from selected districts of Punjab, Pakistan.

Sr.	Districts	GPS Coordinates	Disease incidence (%)	Disease severity (%)
1	Faisalabad	31.4504, 73.1350	31.40	34.57
2	Kasur (Pattoki)	31.0249, 73.8479	51.80	70.00
3	Lahore	31.5204, 74.3587	46.30	41.35
4	Islamabad	33.6844, 73.0479	42.52	59.23
5	Rawalpindi	33.5651, 73.0169	40.19	47..00

DISCUSSIONS

Chlorotic needles and tip dieback followed by wilting are more common and prominent symptoms associated with infected *A. araucana* decline. Landis, 1989 described the same kind of symptoms (wilt, tip dieback, needle discoloration) in pine nurseries. Phylogenetic analyses, macro and microscopic features, and pathogenicity results showed that *Fusarium solani* is the causative agent of *Araucaria araucana* decline in Pakistan. The fungus prevailed throughout the country as a major soil pathogen and was 1st time reported from the northern areas of the country (Armstrong and Armstrong, 1981). Pathogenicity experiment revealed that fungal isolate of *F. solani* recovered from symptomatic tissues of *A. araucana* in Pakistan is pathogenic to *A. araucana*. As the pathogen is soil and seed-borne, it could result in high mortality and severity at juvenile stage and in natural strands. *Fusarium solani* is the most destructive pathogen of tree species (Marin *et al*., 1996; Nelson, 1981**).** The typical physiological (whitish to the creamy colony) and microscopic (aseptate oval-shaped microconidia and septate, curve-shaped macroconidia) characters of the *Fusarium solani* are shared with other *Fusarium* spp. So, this study provides strong evidence of F. solani as the cause of the A. araucana decline. Virulence of Fusarium circinatum in pine nurseries. Furthermore, Pinus sylvestris is highly susceptible to F. solani (Davydenko *et al*., 2018).

The use of antagonists is an effective and eco-friendly approach for the management of fungal pathogen(s). A literature review suggested that Aspergillus and Trichoderma species are widely used as potential antagonists worldwide. So, *A. niger* and *T. harzianum* were selected to determine their potential against pathogenic *F. solani*. *A. niger* and *T. harzianum* showed a significant role in the mycelial suppression of pathogenic *F. solani.* Similar results were obtained with the 50% and 75% concentrations of *A. niger* in the laboratory study against *Fusarium solani* and *Fusarium oxysporum*, the cause of brinjal and tomato wilt (Dwivedi and Enespa, 2013) Aspergillus species are effective antagonists even at their low concentrations against many pathogenic fungi (Gachomo and Kotchoni, 2008). Boughalleb-M'Hamdi *et al*., 2018 reported that *A. niger* was effective against Fusarium solani f. sp. cucurbitae with a per cent inhibition rate above 50%. *Trichoderma* species are involved in the bio-control of several plant pathogens (Harman, 2006). Different isolates of *Trichoderma* were evaluated against *Fusarium solani*, a cause of potato wilt. Among those, *Trichoderma harzianum* provided 76% inhibition of the pathogenic fungus (Ommati and Zaker, 2012). Thus, our results are supported by this study.

In vitro studies of four chemicals (Tebuconazole, Fostyle Aluminum, Thiophanate methyl, Azoxystrobin+ Difenoconazole) suggested Tebuconazole and Thiophanate methyl as effective fungicides. Hence, these chemicals and Salicylic acid were further screened in greenhouse trials. Tebuconazole was highly effective at all concentrations ranging from 50 to 500 ppm in the suppression of Okra's root rot fungus (Fusarium solani) (Kapadiya *et al*., 2013). Baird and Herzog, 1995 reported the effectiveness of Tebuconazole in inhibiting *Fusarium solani*. So, our research findings are under them. Similar results with few variations were obtained during laboratory and field evaluation of Thiophanate methyl and Fostyle Aluminum that significantly inhibited the mycelial growth of *Fusarium solani* (Khanzada *et al*., 2016 and Naik *et al*., 2007). However, SA was most effective compared to Tebuconazole and Thiophanate methyl, which were the least effective. In addition to inhibiting disease severity, SA also enhances the plants' physical appearance.

***Conclusions*:** *Araucaria araucana* is severely affected by the wilt caused by the *F. solani* that was proved as pathogen in this study by completing the Koch's postulates. The fungus was charactreized by morphological and molecular characterization. The characterized isolate showed 100% similarity with *F. solani*. The fungal antagonists, including *Aspergillus niger* and *Trichoderma harzianum*, were evaluated for their antifungal potential. *A. niger* and *T. harzianum* showed 71.1% and 55.2% mycelial inhibition, respectively. Among four tested fungicides, Tiger proved to be the most effective, with 88.8% mycelial suppression. This is 1st comprehensive study on *Fusarium solani* as the etiological agent of *A. araucana* and its management from Punjab, Pakistan.

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***Ethical statement*:** This article does not contain any studies with human participants or animal performed by any of the authors.

***Availability of data and material*:** We declare that the submitted manuscript is our work, which has not been published before and is not currently being considered for publication elsewhere.

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***Code Availability*:** Not applicable

***Authors' contributions*:** Authors 1&2 Plan and execute the research, analyse the data and rest have conducted the experiments and recorded the data.

***Ethics approval*:** This article does not contain any studies with human participants or animal performed by any of the authors.

***Consent to participate*:** All authors are participating in this research study.

***Consent for publication*:** All authors are giving the consent to publish this research article in PDC.

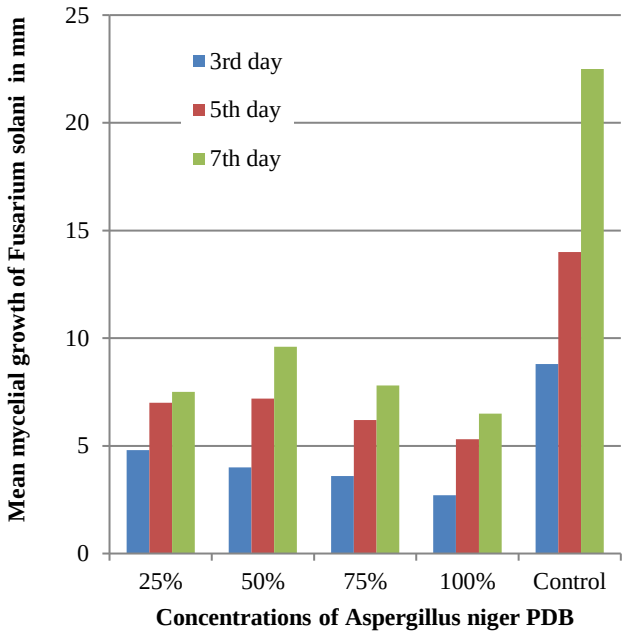

Figure 1. Graphical representation of percent mycelial inhibition of *Fusarium solani* (mm) in control and test plates.

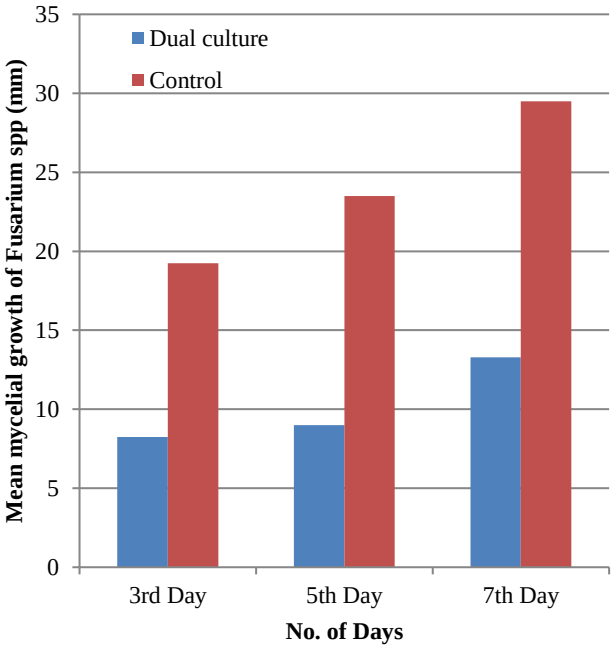

Figure 2. Graphical representation of percent mycelial inhibition of *Fusarium solani* (mm) in comparison with control.

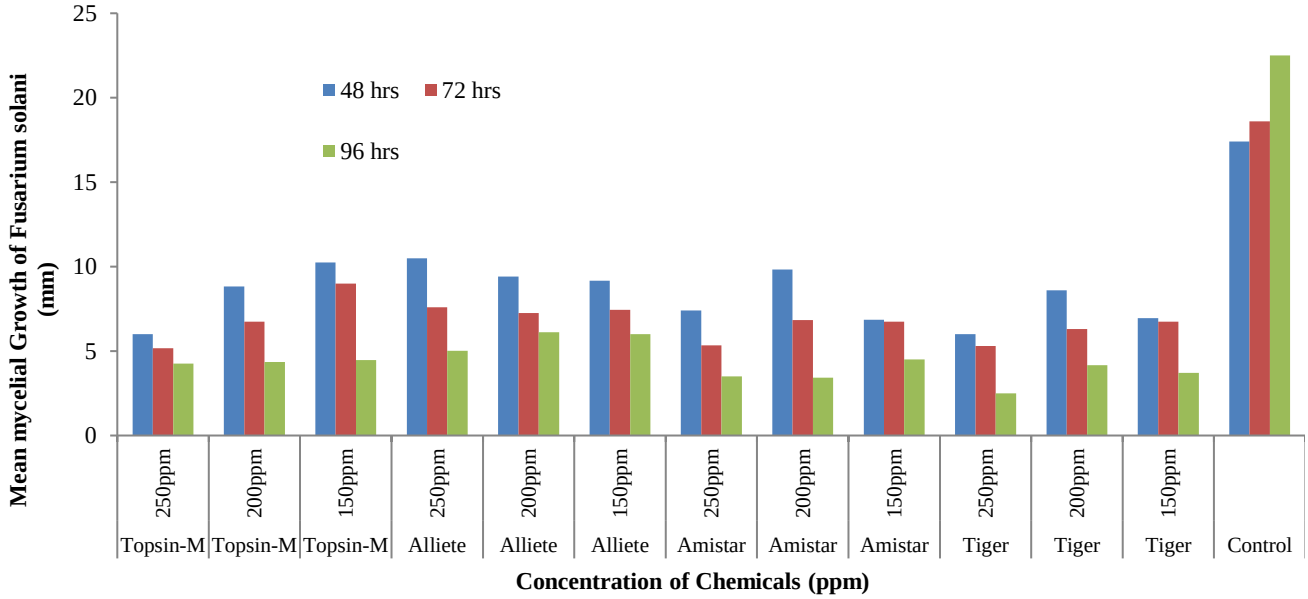

Figure 3. Graphical representation of percent mycelial inhibition of *Fusarium solani* (mm) after 48, 72, and 96 hours at 150, 200, and 250 ppm concentrations.

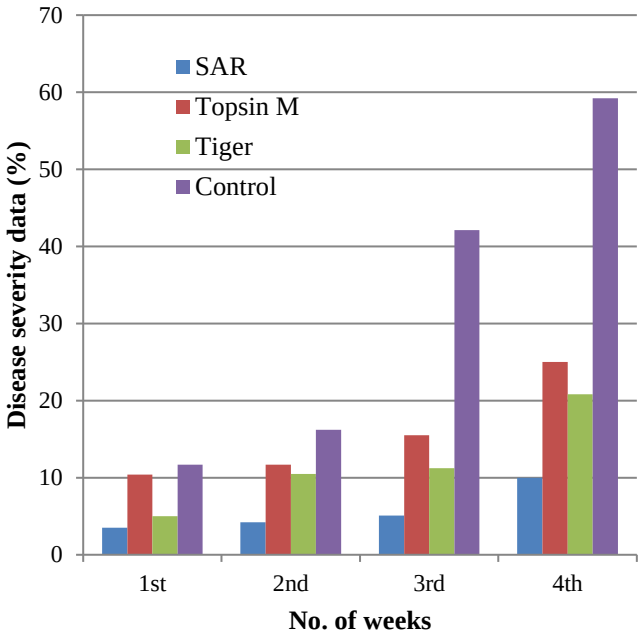

Figure 4.Graphical representation of percent disease severity on salicylic acid and fungicides treated plants in the greenhouse after 1st, 2nd, 3rd, and 4th week.

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